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Article

A new combination of naringin and trimetazidine protect kidney Mitochondria dysfunction induced by renal Ischemia / Reperfusion injury in rat

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Ischemia/reperfusion (IR) injury leads to overproduction of Reactive Oxygen Species (ROS), and disrupts membrane potential that contributes to cell death. The aim of this study was to determine if naringin (NAR), trimetazidine (TMZ) or their combination, protect the kidney mitochondrial from IR injury. Forty rats were randomly allocated into five groups, harboring eight rats each: Sham, IR, NAR (100 mg/kg), TMZ (5 mg/kg) and NAR plus TMZ. Ischemia was induced by obstructing both renal pedicles for 45 min, followed by reperfusion for 4 hours. The mitochondria were isolated to examine the ROS, Malondialdehyde (MDA), Glutathione (GSH), mitochondrial membrane potential (MMP) and mitochondrial viability (MTT). Our findings indicated that IR injury resulted in excessive ROS production, increased MDA levels and decreased GSH, MMP and MMT levels. However, NAR, TMZ or their combination reversed these changes. Interestingly, a higher protection was noted with the combination of both, compared to each drug alone. We speculate that this combination demonstrates a promising process for controlling renal failure, especially with the poor clinical outcome, acquired with NAR alone. This study revealed that pretreatment their combination serves as a promising compound against oxidative stress, leading to suppression of mitochondrial stress pathway and elevation of GSH level.

Keywords: Mitochondria dysfunction. Naringin. Reactive oxygen species. I/R Injury. Trimetazidine.

INTRODUCTION

Ischemia / Reperfusion (IR) is identified as one of causes of acute renal failure (ARF), which is accompanied by mortality and morbidity, and no effective treatment has yet been known for this situation (Bonventre, 1993; Groeneveld *et al*., 1991). It has been previously reported that IR occurs in clinical settings, because of kidney transplantation and hypo-perfusion, followed by circulatory resuscitation or nephrectomy, burns and surgery (Groeneveld *et al*., 1991; Hussein *et al*., 2012). During the ischemic period, mitochondrial membrane permeability (MMP) changes and can produce reactive oxygen species (ROS), subsequently, in the reperfusion phase the generation of ROS is exacerbated, leading to increase in MMP, and reducing mitochondrial antioxidant levels (Jassem *et al.,* 2002; Jassem, Heaton, 2004). Oxidative damage can cause lipid peroxidation, DNA breakdown and could be able to damage mitochondrial proteins, which is considered as different approaches to organ injury (Bonventre, 1993; Bonventre, Yang, 2011). Studies have shown, mitochondrial dysfunction plays a critical role in ROS overproduction (Jassem *et al*., 2002; Jassem, Heaton, 2004). In physiological condition, mitochondrial ROS is neutralized by the mitochondrial antioxidant levels (Jassem, Heaton, 2004). Among the proposed mechanisms most critical one are oxidative stress,

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ROS overproduction and disruption of adenosine triphosphate (ATP) production (Jassem *et al*., 2002). There is a correlation between ROS production from mitochondrial damage and various diseases, including kidney diseases (Sureshbabu, Ryter, Choi, 2015), myocardial injury (Argaud *et al*., 2005) and neurodegenerative diseases (Rao, Carlson, Yan, 2014).

The mitochondrial glutathione (GSH) concentration in the cytosol is similar to that found in the mitochondrial matrix and is identified as the first line of defense against oxidative damage to mitochondrial membranes (Lash, Putt, Matherly, 2002). GSH acts not only as a free radical scavenging substance, but also contributes in several other physiological processes, including cell proliferation, conservation of GSH/GSSG redox, cell signaling, and apoptosis (Mari *et al*., 2009).

Nowadays, antioxidant agents have been used to prevent organ failure in a variety of clinical settings and experimental models (Adil *et al*., 2016; Spargias *et al*., 2004). Naringin (4, 5, 7-trihydroxy flavonone 7- rhamnoglucoside) (NAR) is a substantial natural substance and a polyphenol compound mainly present in grapefruits and citrus species (Chen *et al*., 2016). Among the naturally polyphenols compound, it has been established to have no side effect (Choe *et al*., 2001). Therefore, several studies demonstrated that NAR exhibits, free radical scavenging properties in renal toxicity (Adil *et al*., 2015; Singh, Chander, Chopra, 2004). It has been reported that NAR has a variety of pharmacological characteristics, such as anti-cancer, anti-mutagenic, cholesterol lowering and antioxidant effects, anti-inflammatory, and free radical scavenging effect (Rajadurai, Prince, 2007).

Trimetazidine (TMZ) is known as an anti-ischemic agent (piperazine derivative) that has a protective effect against oxidative stress, because of antioxidant properties and can limit renal IR injury (Nadkarni *et al*., 2015; Sulikowski *et al*., 2008). A recent experimental model study indicated that TMZ can be considered as mPTP (mitochondrial permeability transition pore) inhibitor and could be cardio protective in cardiac IR injury (Argaud *et al*., 2005). The present study highlighted whether the combination of NAR and TMZ could protect kidney mitochondria damage, induced by renal IR injury in a rat model.

MATERIAL AND METHODS

Animals

Forty male Sprague-Dawley rats, weighing 200-250 g, were used and kept in polycarbonate cages at 22 °C, with 50% humidity for 12 hours of light and 12 hours of darkness in an optical cycle. Sufficient water and pellet diet were provided for the rats. Animals are purchased from the Laboratory Animal Reproductive Center of Ahvaz Jundishapur University of Medical Sciences. The experimental protocol was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences.

Chemicals

TMZ, NAR, urethane, rhodamine 123, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), mannitol, ethylene glycol tetra-acetic acid (EGTA), bovine serum albumin (BSA), 2,7-dichlorofluorescein diacetate (DCFH-DA), 3,4 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Coomassie Brilliant Blue, were purchased from Sigma-Aldrich (St Louis, Missouri, USA), and sucrose and dimethyl sulfoxide (DMSO) were obtained from Merck (Darmstadt, Germany).

Experimental design

Forty rats were randomly allocated into five groups of eight rats each. Group I: Sham- operated, all surgical procedures are performed on these animals, except pedicle clamp. Group II: renal IR injury, the animals were given normal saline for 7 days, intraperitoneally, then, surgery and the pedicle clamp technique were done. Group III: the animals received NAR 100 mg/kg, intraperitoneally for 7 days before IR and pedicle clamp method were performed (Gaur, Aggarwal, Kumar, 2009). Group IV: the animals received TMZ 5 mg/kg, intravenously, five minutes before onset of reperfusion

(Cau *et al*., 2008). Group V: rats received TMZ 5 mg/kg, five minutes before beginning of reperfusion plus NAR 100 mg/kg for 7 days before IR. The concentrations of TMZ and NAR were selected in accordance with previous studies and a pilot study (Cau *et al*., 2008; Gaur, Aggarwal, Kumar, 2009).

Induction of IR injury

At the day of surgery, animals were anesthetized by urethane at a dose of 1.7 gr/kg, intraperitoneally (Maleki, Nematbakhsh, 2016). Appropriate anesthetic level was maintained during the test by anesthetic drugs. Animal body temperature was controlled through thermostatic blankets at 37 ° C (Harvard Apparatus, UK). Tracheostomy was performed to ensure airway openness and spontaneous breathing. Midline laparotomy was preformed to expose both of the kidneys. Bilateral ischemia was created by obstructing both renal pedicles, using non-traumatic clamps for 45 min, then, clamps were removed and reperfusion was followed for 4 hours (Nesic *et al*., 2006).

Isolation of kidney mitochondria

Ultimately, after the reperfusion, the kidneys were immediately dissected and placed on the ice plate, then minced with a scissor. Purification buffer solution, containing mannitol (200 mM), HEPES (10 mM), EGTA (1mM), sucrose (70 mM) and 0.1% BSA were added to the kidney and homogenized by homogenizer at 1000 g for 10 minutes. Homogeneous tissue was centrifuged at 4°C, several times at different times, according to the protocol (Hosseini *et al*., 2014). All stages of the isolation and purification of mitochondria were performed on ice. All factors were measured on mitochondrial suspension (0.5 mg protein per ml). Each experiment was repeated 3 times for each rat. Mitochondrial protein level was examined, using the Bradford method (Bradford, 1976).

Determination of mitochondrial membrane potential

The mitochondrial membrane potential (ΔΨm; MMP) collapse was determined, using a cationic fluorescent probe, rhodamine 123, accumulated in the mitochondria by simplifying diffusion and electric gradient force. Healthier mitochondria would collect more rhodamine 123 in their matrices. Aqueous rhodamine solution has an emission peak at 535 nm, whereas matrix and under stacks' rhodamine experiences a fluorescence quenching. When damage occurs, the ratio of red-to-green fluorescence is diminished, compared to the healthy mitochondria. The fluorescence intensity was determined, using a spectrofluorometeric detector (LS50B Perkin Elmer, Waltham, Massachusetts, Ex = 490 nm, Em = 535 nm) (Baracca *et al*., 2003). Our data were expressed as the percentage of mitochondrial membrane potential collapse (%ΔΨm), among groups.

Mitochondrial ROS assay

To measure the amount of ROS, 2′, 7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) was used. DCFH-DA penetrates into mitochondria and hydrolyzes to non-fluorescent DCFH, accumulating in the mitochondria. Then, reaction with ROS, it is oxidized to form highly fluorescent 2, 7-dichlorofluorescein. DCFH-DA was added to the mitochondria suspension (0.5 mg/mL) (Zhang *et al*., 2008). The amount of ROS generation was determined using a spectrofluorometeric detector (UV-1650PC SHIMADZU, Kyoto, Japan), based on the fluorescence intensity unit. The excitation and emission wavelengths are 500 nm and 520 nm, respectively.

Mitochondrial viability (MTT assay)

This colorimetric method is a quantitative measure for viability, in which the yellow salt of tetrazolium is converted to formazan by the mitochondrial dehydrogenase enzyme. MTT solution was added to 1 ml of mitochondrial suspension (0.5 mg/ml), following an incubation period (Mosmann, 1983). Then, 200 µl of DMSO was added and the absorbance was determined at 570 nm wavelength, using a spectrophotometer (UV-1650PC Shimadzu).

Measurement of mitochondria Malondialdehyde (MDA) content

First, 250 µl of 70% trichloroacetic acid was added to the mitochondrial suspension (0.5 mg/mL), and centrifuged at 3000 g for 15 minutes. Then, 0.8% tiobarbituric acid was added to the supernatant, and stood in boiling water for 30 minutes. The absorbance was determined at 532 nm wavelength, using a spectrophotometer (Zhang *et al*., 2008).

Measurement of mitochondrial GSH content

The glutathione mitochondrial content was measured by the formation of yellow color, because of the reaction of glutathione with the indicator of Ellman (DTNB). In summary, mitochondrial suspension was added to 2 ml of Ellman's reagent and the absorbance was read at 412 nm, using a spectrophotometer (Sadegh, Schreck, 2003).

Statistical analysis

Data were analyzed, using SPSS, version 16. Statistical significance was determined using the one-way ANOVA with the Tukey's post hoc test. Statistical significance was considered as $p< 0.05$.

RESULTS

Effect of NAR, TMZ and their combination on MTT assay

MTT as mitochondrial viability assay was determined, concerning the activity of succinate dehydrogenase (complex II). As demonstrated in Figure 1, renal IR injury could cause a remarkable decrease in complex II activity ($p<0.001$), compared to the sham group. However, the results of NAR, TMZ or their combination in pretreatment, indicated a significant increase in MTT level, compared to renal IR injury rats ($p< 0.05$, $p< 0.001$ and $p< 0.001$, respectively) and co-administration revealed a significant increase in MTT level, compared to the NAR alone ($p< 0.01$).

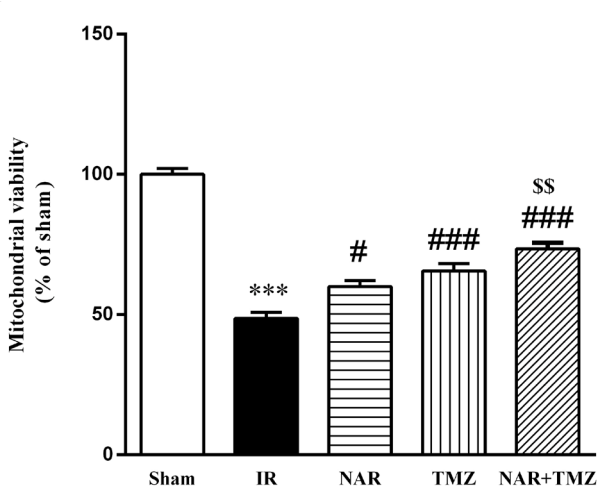

FIGURE 1 - Effect of Naringin (NAR) and Trimetazidine (TMZ) or their combination pretreatment on mitochondrial viability following renal IR injury (mean ± SEM n=8). Sham (sham operated group), IR (Ischemia-reperfusion + normal saline), NAR (Ischemia-reperfusion + 100 mg/kg, intraperitoneally, for one week), TMZ (Ischemia + 5 mg/kg TMZ, intravenously, before reperfusion) and their combination (NAR + TMZ). One-way ANOVA followed by Tukey's post hoc test. *** $p< 0.001$ vs. sham group; # $p< 0.05$; ### $p< 0.001$ vs. IR group; $$ $P< 0.01$ vs. NAR group.

Effect of NAR, TMZ and their combination on MMP

As demonstrated in Figure 2, renal IR injury in kidney led to a significant increase in mitochondrial membrane damage ($p< 0.05$). However, the administration of NAR and TMZ or their combination, reduced the fluorescence intensity, indicating mitochondrial protection. The effects of these drugs result in a significant reduction of damage to MMP ($p< 0.05$, $p< 0.01$ and $p< 0.001$, respectively).

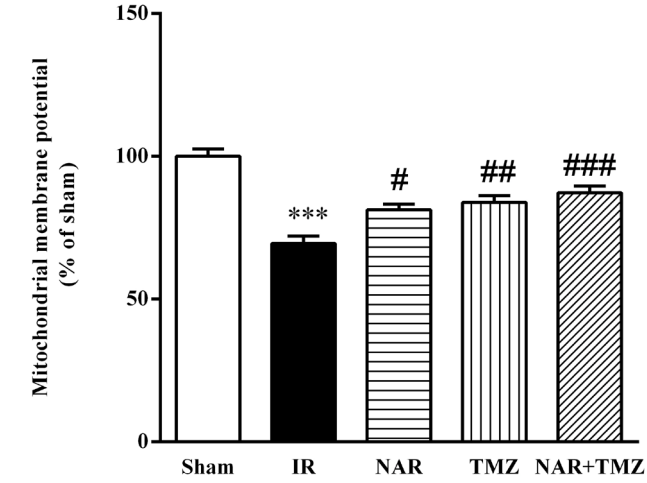

FIGURE 2 - Effect of Naringin (NAR) and Trimetazidine (TMZ) or their combination pretreatment on MMP following renal IR injury (mean ± SEM n=8). Sham (sham operated group), IR (Ischemia-reperfusion + normal saline), NAR (Ischemia-reperfusion + 100 mg/kg, intraperitoneally, for one week), TMZ (Ischemia + 5 mg/kg TMZ, intravenously, before reperfusion) and their combination (NAR + TMZ). One-way ANOVA followed by Tukey's post hoc test. ***p< 0.001 vs. sham group; #p< 0.05; ## p< 0.01; ###p< 0.001 vs. IR group.

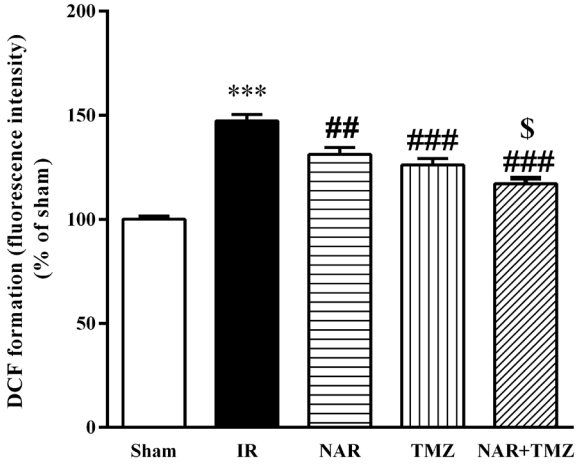

FIGURE 3 - Effect of Naringin (NAR) and Trimetazidine (TMZ) or their combination pretreatment on mitochondrial ROS following renal IR injury (mean ± SEM n=8). Sham (sham operated group), IR (Ischemia-reperfusion + normal saline), NAR (Ischemia-reperfusion + 100 mg/kg, intraperitoneally, for one week), TMZ (Ischemia + 5 mg/kg TMZ, intravenously, before reperfusion) and their combination (NAR + TMZ). One-way ANOVA followed by Tukey's post hoc test. ***p< 0.001 vs. sham group; ## p< 0.01; ###p< 0.001 vs. IR group; $ p< 0.05 vs. NAR group.

Effect of NAR, TMZ and their combination on mitochondria ROS generation

ROS was examined throughout DCFH-DA oxidation. The relative DCF fluorescence intensity demonstrates different ROS concentrations in different groups. Rat's kidney mitochondria (0.5 mg/ml) were isolated, purified, and incubated in buffer, containing sucrose, mannitol, EGTA and HEPES (pH: 7.4) for 1h. As shown in Figure 3, renal IR injury stimulated ROS generation more significantly, compared to the sham group (p< 0.001). Administration of NAR, TMZ or their combination significantly decreased mitochondrial ROS generation, compared to the untreated IR injury group (p< 0.01, p< 0.001, p< 0.001). Moreover, co-administration of NAR and TMZ results in a significant reduction of ROS production, compared to the NAR alone (p< 0.05).

Effect of NAR, TMZ and their combination on the mitochondria lipid peroxidation

As shown in Figure 4, lipid peroxidation was determined by the extent of MDA creation, during an acid heating reaction. In comparison to the sham group, MDA increased remarkably in the IR injury group (p< 0.001). Whereas, TMZ and their combined administration significantly reduced the MDA level, compared to the IR injury group (p< 0.05 and p< 0.001, respectively). Pretreatment with a combination of NAR and TMZ significantly reduced the MDA level, compared to the NAR alone (p< 0.05).

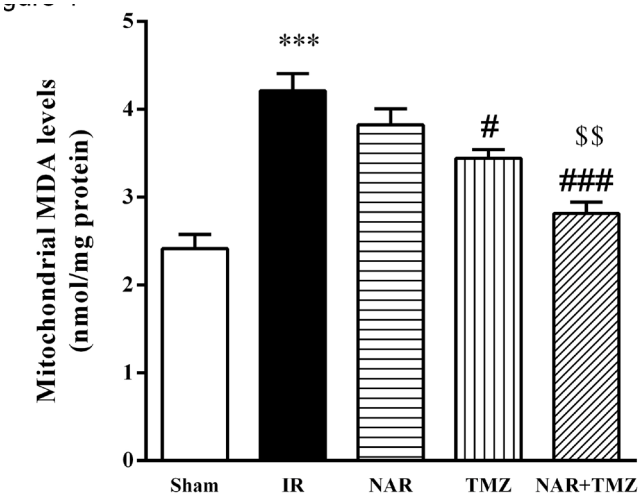

FIGURE 4 - Effect of Naringin (NAR) and Trimetazidine (TMZ) or their combination pretreatment on mitochondrial ROS following renal IR injury (mean ± SEM n=8). Sham (sham operated group), IR (Ischemia-reperfusion + normal saline), NAR (Ischemia-reperfusion + 100 mg/kg, intraperitoneally, for one week), TMZ (Ischemia + 5 mg/kg TMZ, intravenously, before reperfusion) and their combination (NAR + TMZ). One-way ANOVA followed by Tukey's post hoc test. ***p< 0.001 vs. sham group; ## p< 0.01; ###p< 0.001 vs. IR group; $ p< 0.05 vs. NAR group.

Effect of NAR, TMZ and their combination on mitochondria GSH

As shown in Figure 5, a remarkable decrease in mitochondrial GSH levels was seen in the rat IR injury, compared with those in the sham group (p< 0.001). Although, NAR and TMZ alone increased the glutathione content, but this increase was not significant, while their combination improved the depletion of mitochondrial GSH, compared to the renal IR injury group (p< 0.001). Meanwhile, co-administration of NAR and TMZ significantly increased the mitochondrial GSH, compared to either NAR or TMZ alone (p< 0.001). Based on these results, it is recommended that co-administration may provide a crucial role in the elimination of ROS through mitochondrial GSH preservation via GSH synthesis.

Figure 5

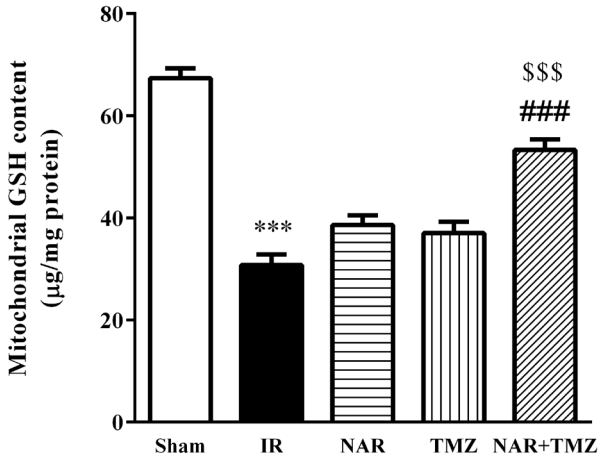

FIGURE 5 - Effect of Naringin (NAR) and Trimetazidine (TMZ) or their combination pretreatment on mitochondrial GSH content following renal IR injury (mean ± SEM n=8). Sham (sham operated group), IR (Ischemia-reperfusion + normal saline), NAR (Ischemia-reperfusion + 100 mg/kg, intraperitoneally, for one week), TMZ (Ischemia + 5 mg/kg TMZ, intravenously, before reperfusion) and their combination (NAR + TMZ). One-way ANOVA followed by Tukey's post hoc test. ***p< 0.001 vs. sham group; ###p< 0.001 vs. IR group; $$$ P< 0.001 vs. NAR or TMZ group.

DISCUSSION

The present data demonstrated that renal IR injury induced kidney mitochondrial oxidative stress via increasing MDA and ROS, and reducing GSH levels. Hence, the findings regarding NAR, TMZ or co-administration of both demonstrated protective effects against oxidative stress induced by IR in isolated kidney mitochondria.

The available evidence suggests that renal IR injury is associated with cellular and molecular events, causing injury to kidney tubular cells (Chatterjee *et al*., 2001; Lieberthal, Levine, 1996). Due to its importance, it necessitates to discover new drugs that are able to protect the kidney from IR injury.

Growing evidence suggest that oxidative stress as a result of ROS generation by renal IR injury, is one of process involved in cell damage and renal dysfunction (Serviddio *et al*., 2008). Mitochondrial ROS is increased under IR injury, hypoxia, toxicity drug and pathologic situation (Ribas, Garcia-Ruiz, Fernandez-Checa, 2014). The mitochondria damage associated with overproduction of ROS, has been identified to play a major role in oxidative stress and cell death (Pieczenik, Neustadt, 2007). Indeed, during IR injury, mitochondria is exposed to alterations that result in diminished ATP generation and overproduction of ROS (Jassem *et al*., 2002). Reperfusion is a necessary stage of ischemic tissue and is associated with the opening of mPTP on the inner mitochondrial membrane, triggering a series of mitochondrial events (Kovacs *et al*., 2010). Opening of mPTP prior to reperfusion time leads to collapse of MMP, after excess production of ROS (Jassem *et al*., 2002). Over generation of ROS led to membrane damage, mPTP opening, and mitochondrial potential disruption (Jassem *et al*., 2002). These results were parallel to what was obtained for ROS generation with IR injury, showing the greatest damage. *In vitro* study in renal proximal cell demonstrated that hypoxia/reoxygenation injury caused mitochondrial stress and antioxidant treatment could reverse this change (Yu *et al*., 2013). The current study showed that renal IR was able to enhance ROS generation in kidney mitochondria. Several studies have reported the beneficial effects of antioxidant enzyme due to the free-radical scavenging properties against AKI-induced IR injury (Hosseini *et al*., 2010; Lee, Son, Kim, 2006). The previous study has shown that NAR could ameliorate mitochondrial dysfunction by reducing oxidative stress (Sachdeva, Kuhad, Chopra, 2014). Experimental study demonstrated that administration of TMZ before ischemia could prevent the reduction of ATP production and disruption of mitochondrial membrane potential, indicating cytoprotective activity against the undesirable effect of IR injury (Elimadi *et al*., 1998).

MDA, a product of polyunsaturated fatty acid peroxidation due to ROS overproduction, is applied as an index of oxidative damage in renal IR injury and multiple diseases (Adil *et al*., 2016; Najafi *et al*., 2015; Visnagri, Kandhare, Bodhankar, 2015). ROS attack the polyunsaturated fatty acid of the cell membrane and cause peroxidation of the membrane lipids, resulting in the loss of membrane integrity and membrane permeability (Chen, Yu, 1994). According to the results of the current study, IR injury result in increasing of mitochondria MDA content, indicating an elevated lipid peroxidation could be related to overproduction of ROS by renal IR. There is evidence that the structure of lipophilic flavonoids leads to interaction with lipid membranes and increases its concentration in the lipid bilayer, suggesting more effective action in scavenging free oxygen radical (Wilcox, Borradaile, Huff, 1999). Evidence from experimental study implicated that NAR produced a significant reduction in MDA level in nephrotoxicity, was inconsistent with the results of the current study (Adil *et al*., 2015). *In vivo* study indicated that TMZ leads to significant decline in lipid peroxidation in renal IR injury (Grekas *et al*., 1996). Interestingly, NAR or TMZ pretreatment caused improvement in lipid peroxidation, however, the best result acquired with co-administration of both.

GSH as a non-enzymatic antioxidant is synthesized by cells and can provide major antioxidant activities against ROS, by its sulfhydryl groups, leading to cellular defense (Ribas, Garcia-Ruiz, Fernandez-Checa, 2014). According to previous studies, the alterations of glutathione metabolism are associated with renal IR injury and different diseases (Mari *et al*., 2009; Zhang *et al*., 2008). In renal proximal tubular cells, it has been reported that the increased content of glutathione has a protecting role against oxidants (Lash, Putt, Matherly, 2002). The experimental study showed that renal IR injury results in oxidative stress and decrease of GSH level in kidney tissue, but the administration of resveratrol prevents the kidney injury through reducing MDA levels and increasing GSH levels (Baltaci *et al.,* 2019).

Based on our data, renal IR injury results in a significant reduction in glutathione level, indicating a decrease in antioxidant capacity, which leads to impairment of antioxidant defense against oxidative stress. The reduction of glutathione content may be related to overproduction of ROS by renal IR injury, further ROS generation can cause oxidization of glutathione pool,

subsequently, result in the formation of protein dithiols, accompanied by antioxidant system deficiency (Ribas, Garcia-Ruiz, Fernandez-Checa, 2014). Administration of antioxidants has been reported to inhibit mitochondria dysfunction through enhancing antioxidant capacity (Ranjbar *et al*., 2018). In this regard, *in vivo* study showed that NAR caused an increase in antioxidant enzymes' activity of the kidney tissue, including superoxide dismutase, catalase and glutathione after renal IR injury model, suggesting NAR's ability to decrease IR induced oxidative damage in the rat kidney (Singh, Chopra, 2004). In experimental and human study, TMZ's ability to increase the glutathione level has been reported (Bayram *et al*., 2005; Suzer *et al*., 2000).

As shown in previous studies, consumption of NAR and TMZ by increasing antioxidant activity and preventing the opening of mPTP (Argaud *et al*., 2005), respectively, have shown to be beneficial against oxidative damage, and their co-administration were more effective than administration of each alone. Recently, we reported that NAR and TMZ or their combination had a renoprotective effect through increasing antioxidant capacity and inhibition of apoptosis signaling that result in improvement of kidney function (Amini *et al*., 2019a). In another study indicated that NAR and TMZ or their combination inhibit myocardial injury in renal IR injury model via enhancing the Nrf-2 expression (Amini *et al*., 2019b).

CONCLUSION

In summary, NAR or TMZ could significantly inhibit IR-induced oxidative stress, therefore preserving the kidney mitochondria. Higher safeguarding was proposed by a combination of TMZ and NAR. Consequently, we speculate that this mixture demonstrates a promising process during AKI, especially one caused by IR injury, suggesting a renoprotective effect against oxidative stress, induced by IR injury.

CONFLICT OF INTERESTS

The authors declare that have no conflict of interest.

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